

**Operation of a sequencing batch reactor for cultivating autotrophic nitrifying  
granules**

Xian-Yang Shi<sup>1,2</sup>, Guo-Ping Sheng<sup>1,\*</sup>, Xiao-Yan Li<sup>3</sup>, Han-Qing Yu<sup>1,\*\*</sup>

<sup>1</sup>Department of Chemistry, University of Science & Technology of China, Hefei,  
230026 China

<sup>2</sup>Institute of Life Sciences, Anhui University, Hefei 230039, China

<sup>3</sup>Department of Civil Engineering, University of Hong Kong, Pokfulam Road, Hong  
Kong

**Corresponding authors:**

Guo-Ping Sheng, Fax: +86 551 3601592; E-mail: [gpsheng@ustc.edu.cn](mailto:gpsheng@ustc.edu.cn)

Han-Qing Yu, Fax: +86 551 3601592; E-mail: [hqyu@ustc.edu.cn](mailto:hqyu@ustc.edu.cn)

## Abstract

The granulation of nitrifying sludge in a sequencing batch reactor (SBR) fed with  $\text{NH}_4^+$ -N-laden inorganic wastewater was investigated. After 120-day operation spherical and elliptical granules with an average diameter of 0.32 mm were observed. The hydrophobicity surface, settling velocity and specific gravity of the matured granules increased with the processing of sludge granulation. Spatial distribution of bacterial species within the autotrophic granules was analyzed with fluorescence in situ hybridization. Both ammonia- and nitrite-oxidizing bacteria were observed in the granular sludge. The Michaelis-Menten equation was used to describe their  $\text{NH}_4^+$ -N utilization rate, and the kinetic coefficients were calculated to be  $v_m = 18.0$  mg/g-VSS/h and  $K_m = 36.7$  mg/l. Taking into account the  $\text{NH}_4^+$ -N utilization rate and removal efficiency together, an  $\text{NH}_4^+$ -N concentration range of 100-250 mg/l was found to be favourable for the operation of the SBR to cultivate nitrifying granules.

**Keywords:** Aerobic granule; Ammonia-oxidizing bacteria (AOB); Autotrophic; Kinetics; Nitrite oxidizing bacteria (NOB)

## 1. Introduction

Nitrogen compounds like ammonia and nitrate can be found in many wastewaters and need to be removed in order to prevent oxygen depletion and eutrophication of surface waters. Nitrification, the biological oxidation of ammonia, was described already a century ago. Extensive reviews on autotrophic nitrification and nitrogen

1 removal from wastewater are available (van Benthum et al., 1996). The  
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3 rate-determining step in this process is nitrification, which is accomplished by  
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5 autotrophic nitrifying bacteria under aerobic conduction (Ruiz et al., 2003; Ni et al.,  
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7 2008). It is difficult to obtain and maintain sufficient nitrifying bacteria in wastewater  
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9 treatment plants due to their very low growth rates.  
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14 In order to solve the problem, various techniques for retaining nitrifying bacteria  
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16 with high density in a reactor have been recently proposed, e.g., entrapment in a  
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18 hydrogel matrix of polyvinyl alcohol (Myoga et al., 1991) or polyethylene glycol  
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20 (Sumino et al., 1992; Isaka et al., 2007). However, development of a simpler and  
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22 more effective immobilization method for nitrifying bacteria is still demanded.  
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27 Aerobic granulation represents an innovative cell immobilization strategy in  
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29 biological wastewater treatment and it is attracting increasing interests (Beun et al.,  
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31 1999; Zheng et al., 2005; Su and Yu, 2005; Wang et al., 2007; Liu et al., 2009).  
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36 Aerobic granules are self-immobilized microbial aggregates that are usually cultivated  
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38 in sequencing batch reactors (SBR) without adding a carrier material. Many  
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40 researchers reported aerobic granulation for efficient treatment of organic wastewater  
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42 (Zheng et al., 2005; Su and Yu, 2005; Wang et al., 2007). However, the information  
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44 on the nitrifying bacteria granulation with inorganic wastewater rich in ammonium is  
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46 limited (Tsuneda et al., 2003; Liu et al., 2008; Ni and Yu, 2008). Tsuneda et al. proved  
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48 that nitrifying bacteria could be self-immobilized in an aerobic upflow fluidized  
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50 reactor. Spherical, pseudocubic and elliptical granules with a diameter of 0.35 mm  
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52 were produced at the bottom of the reactor after 300 days of operation. The reactor  
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was operated continuously at a hydraulic retention (HRT) of 7.6 h and influent  $\text{NH}_4^+\text{-N}$  of 500 mg/l. Liu et al. also cultivated nitrifying granules in an SBR (Tsuneda et al., 2003). Although autotrophic nitrifying granules for nitrification have been developed, the formation of nitrifying granules and the distribution of ammonia-oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in the nitrifying granules are still not clear up to now yet.

Therefore, the main objective of this work was to cultivate nitrifying granules in an SBR and elucidate the distribution of AOB and NOB in the granules. The physical properties of granules, such as hydrophobicity of cell surface, settling velocity and specific gravity, were also investigated. It is expected that the work would be useful to better understand the mechanisms responsible for the granulation of nitrifying cultures and apply them for the treatment of  $\text{NH}_4^+\text{-N}$ -laden inorganic wastewaters.

## **2. Materials and methods**

### *2.1. Reactor set-up and operation*

The SBR had a working volume of 3.4 l with an internal diameter 6.0 cm and a height of 130.0 cm. The reactor was operated for 6 h each circle with a HRT of 12 h. Effluent was drawn at 60.0 cm from the bottom, resulting in 1.7 l of mixed liquor left in the reactor after effluent withdrawal. The filling and withdraw time were 2 min and 5 min respectively. The settling time was varied from 20 to 5 min and the remainder

was the reaction time. The seeding sludge, taken from an aeration tank in Wangxiaoying Municipal Wastewater Treatment Plant, Hefei, China, was pre-cultivated in a batch reactor supplied with ammonium and inorganic carbon for 6 weeks. The pre-cultivated sludge had a mixed liquor suspended solids (MLSS) concentration of 12.0 g/l and a sludge volume index (SVI) of 42.8 ml/g. Sludge of 1.7 l was inoculated into the SBR, resulting in an initial MLSS concentration of 7.0 g/l in the reactor. The experiment was performed in a temperature-controlled room at  $25\pm 1^{\circ}\text{C}$ . Air was introduced through an air diffuser by an air pump at the bottom of the reactor.

A synthetic wastewater was used based on the previous studies (Tsuneda et al., 2003; Liu et al., 2008). This wastewater, with a similar characteristics to tannery wastewater (Carrera et al., 2003) , had the following compositions:  $\text{NH}_4\text{Cl}$ , 764 mg/l;  $\text{NaHCO}_3$ , 2200 mg/l;  $\text{MgSO}_4$ , 25 mg/l;  $\text{FeSO}_4$ , 5 mg/l;  $\text{CaCl}_2$ , 5 mg/l and microelement solution 1.0 ml/l. To satisfy the growth requirement of nitrifying bacteria, the ratio (w:w) of bicarbonate to ammonium-nitrogen was kept over 8.0. The microelement solution contained:  $\text{H}_3\text{BO}_3$ , 0.15 mg/l;  $\text{ZnCl}_2$ , 0.05 mg/l;  $\text{CuCl}_2$ , 0.02 mg/l;  $\text{MnSO}_4\cdot\text{H}_2\text{O}$ , 0.05 mg/l;  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ , 0.06 mg/l;  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ , 0.15 mg/l;  $\text{FeCl}_3$ , 0.05 mg/l and  $\text{NiCl}_2$ , 0.04 mg/l.

## 2.2. Identification of nitrifying bacteria composition

The granule samples, taken from the reactors on the 180<sup>th</sup> day, were fixed in 4% freshly prepared paraformaldehyde solution for 6 h at  $4^{\circ}\text{C}$  and then washed twice with

phosphate-buffered saline (PBS). The granules were then exposed to 50% ethanol in PBS for 12 h at -20°C. The fixed granules were dehydrated by successive passages through 50, 80, and 100% ethanol (three times), 50:50 (vol/vol) ethanol-tert-butyl alcohol, and 100% tert-butyl alcohol (three times) and embedded in melted paraffin wax. The sections of 20 µm thick were cut with a rotary microtome and mounted on gelatin-coated glass slides. The sections were dewaxed through 100% xylene (two times) and 100% ethanol (two times). After air drying at room temperature, hybridization was conducted following the established method (Sekiguchi et al., 1999).

A ROX-labeled NSO190 probe (5'CGATCCCCTGCTT TTCTCC3') targeting AOB and a FITC-labeled NIT3 probe (5'CCTGTGCTCCATGCTCCG3') targeting *Nitrobacter* were used. The hybridization image was captured using a fluorescence microscope (Leica, DM6000B). For quantitative analysis of FISH images, about 10 images were scanned and averaged by image processing software (IMT i-Solution, version 3.0).

### 2.3. Analysis

The MLVSS and SVI were measured according to the Standard Methods (APHA, 1998). Ammonium, nitrate and nitrite concentrations were determined colorimetrically following the Standard Methods (APHA, 1998). Changes in morphology of the granules, size, specific gravity, cell hydrophobicity and settling velocity were determined according to the methods reported by Su and Yu (2005).

### 3. Results and discussion

#### 3.1. Formation of nitrifying granules

The seeding sludge with a mean floc size of 0.10 mm had a fluffy, irregular and loose-structured morphology. After 120-day operation, spherical and elliptical granules were formed. These granules increased in size and their average diameters reached 0.32 mm. The nitrifying granules had a compact and round-shaped structure with a clear outer shape. No filamentous bacteria were observed on the granule surfaces.

The aerobic granulation, i.e., from dispersed sludge to mature granules, is a gradual and slow process. Previous studies showed that the settling time had a significant influence on the aerobic granulation and that a short settling time was favourable for the granule formation (Lei et al., 2004). The settling time required for successful aerobic granulation would not be longer than 5 min. However, for a too short settling time a large volume of nitrifying population could not be maintained efficiently for the granulation because of their low growth. Thus, the settling time of the SBR was gradually decreased from 20 to 8 min. When the biomass concentration and SVI reached a pseudo-steady state, the settling time was fixed at 8 min. The changing patterns of MLVSS and SVI in the continuous operation of the SBR are

illustrated in Fig. 1.

After seeding, the biomass concentration in term of MLVSS in the reactor decreased gradually because some microorganisms with poor setting properties were washed out of the reactor, and reached a relatively stable level of about 2.0 g/l on day 40 (Fig. 1). The initial SVI of seeding sludge was 42.8 ml/g. As shown in Fig. 1, the SVI first gradually increased and then decreased. At the end of experiment, the SVI decreased to only 36.4 ml/g, suggesting that the mature granular sludge had a more excellent settling capacity compared with the seeding sludge.

It took a long time period for the nitrifying bacteria with low growth rates to become granulation. Washout of flocs from an SBR is one of the essential strategies for aerobic granulation. Suitable aeration volume and hydrodynamic shear force are usually favorable to promote the nitrifying granulation (Tay et al., 2001). The present study demonstrates that at an HRT of 12 h, an air flow of 3.0 l/min, settling time of 8 min and a load of 0.235 kg  $\text{NH}_4^+ \text{-N}$  /l/d were appropriate to the formation of nitrifying granules.

### 3.2. Comparison between seed sludge and nitrifying granules

Table 1 summarizes the characteristics of the seeding sludge and the nitrifying granules at the end of experiment. The specific gravity of sludge increased from 1.005 g/cm<sup>3</sup> at the beginning of the experiment to 1.018 g/cm<sup>3</sup>. Such a significant improvement of specific gravity indicates their highly compact structure. The



1 decrease in SVI and the increase in settling velocity clearly show that the sludge  
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3 settling ability improved considerably as the granulation progressed.  
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6 Hydrophobicity of cell surface is considered to play an important role in the  
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8 self-immobilization and attachment of cells to a surface. The seeding sludge had a  
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10 mean contact angle value of 35.4° and increased to 72.8° on day 180. A significant  
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12 difference in cell hydrophobicity between the seeding sludge and the nitrifying  
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14 granules reveals that the formation of the nitrifying granules was coupled to an  
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16 increase in the cell surface hydrophobicity. Similar results have been also found in  
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18 other studies (Tay et al., 2001; Zheng et al., 2005).  
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### 28 3.3. FISH image analysis of nitrifying bacteria 29 30 31 32 33

34 **Figure 2** illustrates the FISH images of the nitrifying granules collected from the  
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36 SBR on day 180. The nitrifying granules were simultaneously hybridized with  
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38 NSO190, AOB domain specific probe, labeled with ROX (Fig. 2b) and NIT3,  
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40 *Nitrobacter* domain specific probe, labeled with FITC (Fig. 2c). The FISH images  
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42 (Fig. 2a) illustrate that the AOB were found close to the granule surface, while that  
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44 the NOB, i.e., *Nitrobacter*, were found in the deeper layer of granules, which might  
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46 take advantage of the product (nitrite) formed by the AOB. Quantitative FISH image  
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48 analyses of samples on day 180 show that the AOB occupied 62.7-63.6% in the total  
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50 bacteria, while *Nitrobacter* occupied only 14.8-15.5%.  
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58 A typical changing pattern of  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$  and  $\text{NO}_3^-\text{-N}$  concentrations **in one**  
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operating cycle of the SBR is illustrated in Fig. 3. The  $\text{NH}_4^+$ -N concentration decreased rapidly and input  $\text{NH}_4^+$ -N was converted to nitrite and nitrate. Furthermore, a temporary nitrite accumulation with respect to nitrate formation was observed, indicating that activities of both AOB and NOB in granules were high. The results without nitrite accumulation suggest that, in addition to *Nitrobacter*, other NOB might also be present in the granules, converting nitrite to nitrate. Recently, *Nitrospira* has been found to play a role in nitrite oxidation in engineered systems (Kim and Kim, 2006). Coskuner and Curtis (2002) reported that *Nitrospira* and *Nitrobacter* could be coexisted in a full scale activated sludge plant. Thus, both *Nitrospira* and *Nitrobacter* might be present in the nitrifying granules.

The maximum number of the AOB and *Nitrobacter* occupied 79.1% of the total bacteria, suggesting that other AOB, NOB and heterotrophs might exist in nitrifying granules, despite of no organic matter in the feeding solution. Extracellular polymeric substances, produced by these heterotrophs, could be favorable to nitrifying granulation through stabilizing the scaffold of the granule and maintaining the three-dimensional structure (Tsuneda et al., 2003).

### 3.4. Kinetics of substrate utilization

Nitrifying bacteria are strictly aerobe, sensitive to substrate concentration and other operating conditions such as temperature and pH. In order to investigate the effects of substrate concentration on nitrification,  $\text{NH}_4^+$ -N concentration in the feeding

solution was varied from 50, 100, 150, 200, and 250 to 300 mg/l. At each  $\text{NH}_4^+\text{-N}$  concentration, the SBR was operated for 2 weeks. For this SBR, its  $\text{NH}_4^+\text{-N}$  utilization rate is directly related to the nitrifying bacteria concentration in the granules and the  $\text{NH}_4^+\text{-N}$  concentration surrounding the granules. The  $\text{NH}_4^+\text{-N}$  concentrations of influent and effluent (after reaction for 2 h) were examined and recorded as  $S_o$  and  $S_e$  respectively. The substrate utilization rate is ratio of the difference of  $S_o$  and  $S_e$  and the sludge concentration within 2 h. Fig. 4 shows the nitrifying rate increased rapidly as the  $\text{NH}_4^+\text{-N}$  concentration was increased from 50 to 200 mg/l and remained constant from 250 to 300 mg/l. Variations of the  $\text{NH}_4^+\text{-N}$  removal efficiency with the influent  $\text{NH}_4^+\text{-N}$  concentration are also illustrated in Fig. 4. On the contrary, an increase in  $\text{NH}_4^+\text{-N}$  concentration resulted in a decrease in  $\text{NH}_4^+\text{-N}$  removal efficiency.

Furthermore, the Michaelis-Menten equation was used to model the  $\text{NH}_4^+\text{-N}$  utilization as follows (Wu et al., 2002):

$$v = \frac{v_m S}{K_m + S} \quad (1)$$

where  $v$  is the  $\text{NH}_4^+\text{-N}$  utilization rate (mg/g-VSS/ h),  $v_m$  is the maximum  $\text{NH}_4^+\text{-N}$  utilization rate,  $K_m$  is the dissociation constant (mg/l) and  $S$  is the  $\text{NH}_4^+\text{-N}$  concentration. The double reciprocal form of Eq. (1) can be given:

$$\frac{1}{v} = \frac{K_m}{v_m} \frac{1}{S} + \frac{1}{v_m} \quad (2)$$

Plotting  $1/v$  against  $1/S$ , a straight line was obtained with an intercept of  $1/v_m$  and a slope of  $K_m/v_m$ . From the slope and intercept of the best-fit line,  $v_m$  and  $K_m$  could be

estimated and thus the kinetics equation of substrate utilization was determined. According to Eqs. (1) and (2), with a plot  $1/v$  against  $1/S$ , the values of  $v_m$  and  $K_m$  were estimated as 18.0 mg/g-VSS/h and 36.7 mg/l, respectively. The regression line had a correlation coefficient of 0.987 implying the applicability of Eq. (1). Therefore, the kinetic equation of the  $\text{NH}_4^+$  utilization was:

$$v = \frac{S}{2.0343 + 0.055S} \quad (3)$$

The  $K_m$  value represents the  $\text{NH}_4^+$ -N level required to reach 50% of the maximum  $\text{NH}_4^+$ -N utilization rate and could be used for adjusting the most appropriate  $\text{NH}_4^+$ -N level. The  $v_m$  value of 18.00 mg/g-VSS/h is significantly higher than a value of 3.29 mg g-VSS/h in an airlift reactor as reported by Carvalho et al. (2002). This difference suggests that the nitrifying granules cultivated in the present work had a high nitrification rate. Thus, taking into account both  $\text{NH}_4^+$ -N utilization rate and removal efficiency together, an  $\text{NH}_4^+$ -N concentration range of 100-250 mg/l was appropriate for the effective operation of the SBR with nitrifying granules.

#### 4. Conclusions

- After 120-day of operation, compact nitrifying granules were formed. Their surface hydrophobicity, settling velocity and specific gravity increased with the sludge granulation.
- The AOB were close to the granule surface, while the NOB were found in the

deeper layer of granules. The total number of *Nitrobacter* was much smaller than that of the AOB.

- The Michaelis-Menten equation could appropriately describe the  $\text{NH}_4^+$ -N utilization rate of the granules with a  $v_m$  of 18.0 mg/g-VSS/h and  $K_m$  of 36.7 mg/l.
- An  $\text{NH}_4^+$ -N of 100-250 mg/l was found to be appropriate for the operation of the nitrifying SBR.

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Table 1 Characteristics of the seeding sludge and the nitrifying granules

Item	Seeding sludge	Nitrifying granules
MLVSS (g/l)	6.0±0.3	2.3±0.2
MLVSS/MLSS (%)	70.0±4.5	81.9±5.3
SVI (ml/g)	92.0±4.3	36.4±2.1
Average diameter (mm)	<0.1	0.323±0.018
Settling velocity (m/h)	<1.0	1.7-2.8±0.5
Specific gravity (g/cm <sup>3</sup> )	1.005±0.001	1.018±0.003
Contact angle	35.4°±3.5	70.2°±4.8

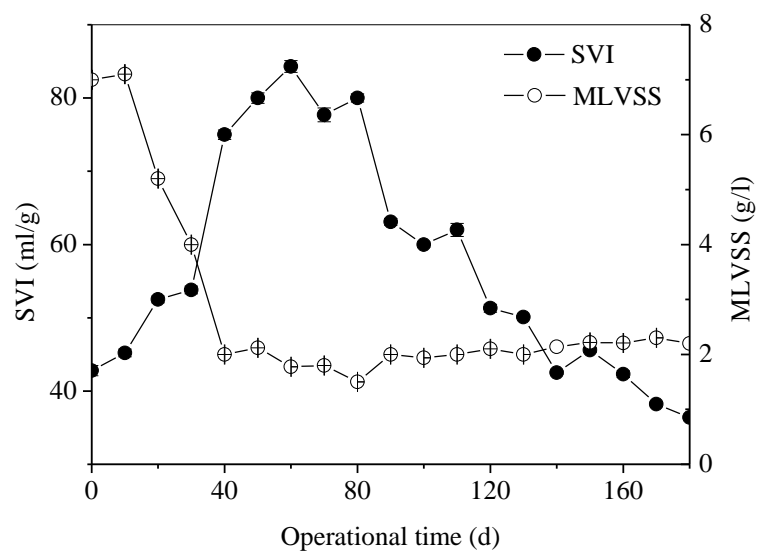
### Figure captions

Figure 1 Changing patterns of SVI and MLVSS in the continuous operation of the SBR.

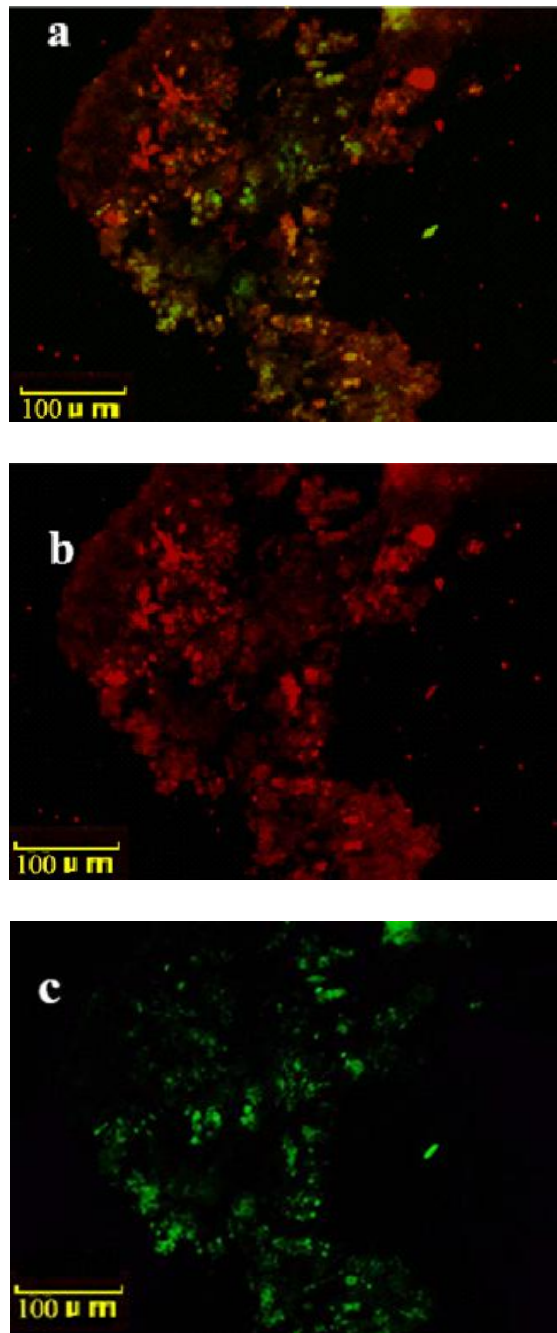
Figure 2 FISH images of aerobic granules on 180<sup>th</sup> day. (a) ROX-labeled probe NSO190 (red) and FITC-labeled probe NIT3 (green); (b) ROX-labeled probe NSO190; and (c) FITC-labeled probe NIT3.

Figure 3 Nitrification profiles observed in a cycle phase

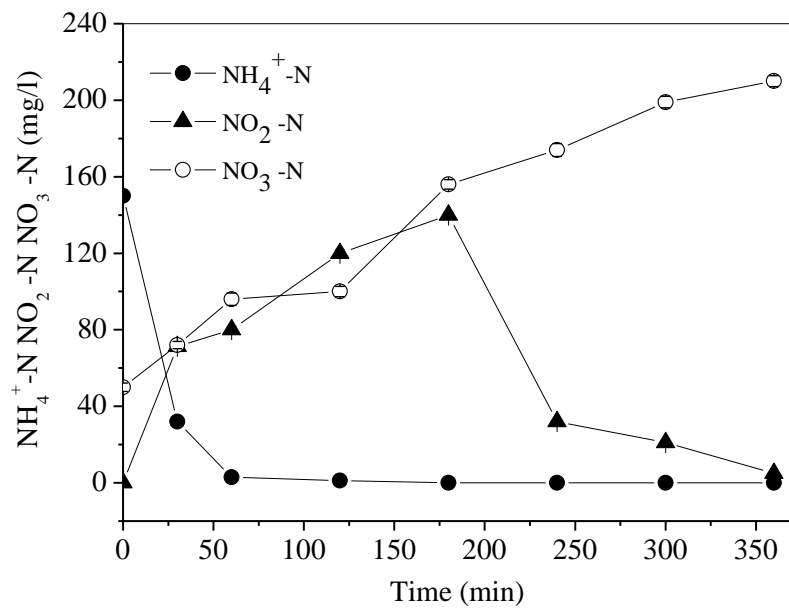
Figure 4 Variations of the substrate utilization rate and  $\text{NH}_4^+$ -N removal efficiency at different influent  $\text{NH}_4^+$ -N concentrations: ● substrate utilization rate, and ○  $\text{NH}_4^+$ -N removal efficiency.



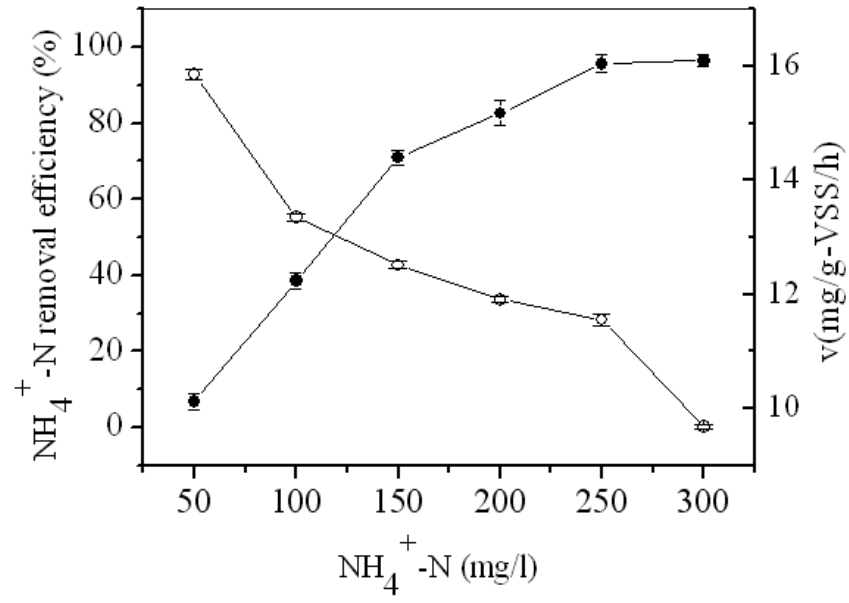
**Fig. 1.** Changing patterns of SVI and MLVSS in the continuous operation of the SBR



**Fig. 2.** FISH images of aerobic granules on 180<sup>th</sup> day. (a) ROX-labeled probe NSO190 (red) and FITC-labeled probe NIT3 (green); (b) ROX-labeled probe NSO190; and (c) FITC-labeled probe NIT3



**Fig. 3.** Nitrification profiles observed in a cycle phase



**Fig. 4.** Variations of  $\text{NH}_4^+\text{-N}$  removal efficiency and the substrate utilization rate at different influent  $\text{NH}_4^+\text{-N}$  concentrations: ● the substrate utilization rate, and ○  $\text{NH}_4^+\text{-N}$  removal efficiency